

The Relationship of Surface Characteristics and Antimicrobial Performance of Pulp Capping Materials

Farrugia, Cher; Lung, Christie Y K; Schembri Wismayer, Pierre; Arias-Moliz, Maria Teresa; Camilleri, Josette

DOI:

[10.1016/j.joen.2018.04.002](https://doi.org/10.1016/j.joen.2018.04.002)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Farrugia, C, Lung, CYK, Schembri Wismayer, P, Arias-Moliz, MT & Camilleri, J 2018, 'The Relationship of Surface Characteristics and Antimicrobial Performance of Pulp Capping Materials', *Journal of Endodontics*.
<https://doi.org/10.1016/j.joen.2018.04.002>

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Manuscript Number: JOE 17-646R1

Title: The relationship of surface characteristics and antimicrobial performance of pulp capping materials

Article Type: Regenerative Endo. (clinical or basic)

Section/Category: Regenerative Endodontics

Keywords: Surface micro-morphology, antimicrobial activity, light curable pulp capping materials

Corresponding Author: Professor josette camilleri,

Corresponding Author's Institution: University of Birmingham

First Author: cher Farrugia

Order of Authors: cher Farrugia; Christie Y Lung; Pierre Schembri Wismayer ; Maria Teresa Arias Moliz; josette camilleri

Manuscript Region of Origin: Europe & Central Asia

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Author names and affiliations.

Cher Farrugia^a, Christie YK Lung^b, Pierre Schembri Wismayer^c, Maria Teresa Arias Moliz^d,
Josette Camilleri^{a,e}

^a*Department of Restorative Dentistry, Faculty of Dental Surgery, University of Malta, Malta;*
cherfarrugia@gmail.com; ^b*Dental Materials Science, Faculty of Dentistry, The University of*
Hong Kong, Hong Kong; yklung@graduate.hku.hk; ^c*Department of Anatomy, Faculty of*
Medicine and Surgery, University of Malta, Malta; pierre.schembri-wismayer@um.edu.mt;
^d*Faculty of Dentistry, University of Granada, Granada, Spain;* mtarias@ugr.es, ^e*School of*
Dentistry, College of Medical and Dental Sciences, Institute of Clinical Sciences, University of
Birmingham, Birmingham, United Kingdom; J.Camilleri@bham.ac.uk

Contribution of each author: Farrugia C.: Microbiology, writing and editing manuscript;
Lung C.: material creator; Schembri Wismayer P.: Laboratory work, editing manuscript; Arias
Moliz MT: Microbiology, protocols, writing and editing manuscript; Camilleri J.:
Characterization, writing, editing, finalizing manuscript and originator of idea.

Corresponding author:

J. Camilleri
School of Dentistry
College of Medical and Dental Sciences
Institute of Clinical Sciences
University of Birmingham
Birmingham
United Kingdom
J.Camilleri@bham.ac.uk

Acknowledgement

The authors deny any conflicts of interest

The relationship of surface characteristics and antimicrobial performance of pulp capping materials

Abstract:

Introduction: Pulp capping materials need to be able to protect the pulp but also bond to the overlying restorative materials. Light curable pulp capping materials bond better to restorative materials and are easier to place than most water-based cements. The aim of this study was to characterize new light curable tricalcium silicate-based pulp capping materials and compare their surface and antimicrobial properties to clinically available Theracal and Biodentine.

Methods: The surface characteristics of three light curable pulp capping materials based on a resin and filled with tricalcium silicate and tantalum oxide radiopacifier, and Theracal and Biodentine were assessed by scanning electron microscopy, X-ray diffraction and contact angle measurement. The radiopacity was measured following ISO 6876 standards. The antimicrobial activity was determined by direct contact test and antibiofilm activity by adenosine triphosphate (ATP) assay and confocal laser scanning LIVE/DEAD ® assay using a polymicrobial culture.

Results: Surface characteristics of the materials varied with the unfilled resin and Biodentine exhibiting a hydrophobic surface. Biodentine showed significantly higher antimicrobial properties in the direct contact test, but this property was absent in the antibiofilm activity tests. The resins filled with tricalcium silicate and Theracal showed higher antimicrobial activity than Biodentine in the ATP and LIVE/DEAD ® assays.

Conclusions: Surface characteristics of a material affect its antimicrobial properties. The experimental resin modified materials exhibited comparable antimicrobial properties to other light curable pulp capping agents. Further long term studies on the

materials' antimicrobial activity are required to assess whether they can result in better clinical outcomes.

Keywords:

Surface micro-morphology, antimicrobial activity, light curable pulp capping materials

Introduction

Pulp capping materials are used to seal pulp wounds, facilitate dentine bridge formation and as a result preserve the dental pulp and prevent root canal therapy (1). Ideal pulp capping materials should induce dentin bridge formation and possess antibacterial properties (2), but many of the commercially available materials do not satisfy clinical requirements. With current pulp capping systems, micro-gaps may be present at the pulp capping material to dentine interface, as well as between the pulp capping material and the composite restorative material (3), potentially resulting in failure of the interface.

Mineral trioxide aggregate (MTA) is a tricalcium silicate-based material that has shown greater dentine bridge formation than the traditionally used calcium hydroxide. However, MTA-based materials are generally difficult to handle and have an extended setting time (4), making them clinically less attractive than materials which have controlled setting properties. Furthermore, bonding of a composite restoration to MTA may be problematic (5,6).

Theracal (7,8) and Biodentine (9) have been extensively studied in the literature as possible alternatives for pulp capping. Theracal provided a more clinically efficient solution due to its light curing properties, but has shown less calcium ion release than Biodentine (10,11). Biodentine exhibits good results both in *in vitro* testing and clinically, due to its easier handling properties and biocompatibility but still demonstrates problems to bond to composite (12,13) and etching of Biodentine results in deterioration of material properties and microleakage. A delay in placement of final restoration when using Biodentine is advised (7,14).

The aim of this study was to characterize new light curable resin-based pulp capping materials. The study compares the antimicrobial properties of the novel materials to those of two commercially available products using a polymicrobial biofilm.

Methodology

The materials investigated in this study included the following:

- Experimental light curing resin (LC)
- Experimental light curing resin with tricalcium silicate filler in 60:40 proportion by volume (LC-TCS)
- Experimental light curing resin with tricalcium silicate filler in 60:40 proportion by volume radiopacified with tantalum oxide replacing the filler by 20% by volume (LC-TCS-TO)
- [Theracal](#) (Bisco, Schaumburg, IL, USA)
- Biodentine (Septodont, Saint-Maur-des-Fossés, France)

The experimental resins have been described in a previous publication (15).

Dosage by volume was used for the experimental materials to determine the percentage replacement of the filler and radiopacifier since these had different densities thus dosage by weight would have led to inaccuracies. [Experimental samples were light cured for 20 seconds using a LED curing light \(Bluepahse 20i \(Ivoclar Vivadent, Mississauga, Canada\) with an approximately wavelength of 470 nm.](#) The materials were cured in 1mm increments to allow the curing light to penetrate the deeper layers. The Theracal and Biodentine were prepared following the manufacturer instructions.

Material characterization

After setting, the materials were stored in Hank's balanced salt solution (HBSS; Sigma Aldrich, Gillingham UK) for 24 hours after which they were retrieved, dried and characterized. The surface micro-morphology was assessed by scanning electron microscopy (SEM). Phase analysis was performed by glancing angle X-ray diffraction analysis at a fixed angle of incidence of 3° over a solid surface. The X-ray diffractometer (Rigaku, Tokyo, Japan) was operated in grazing-incidence asymmetric Bragg mode using Cu K α radiation, an operating current of 40 mA and voltage of 45 kV for $15-45^\circ 2\theta$ with a sampling width 0.05° , scan speed $0.8^\circ/\text{min}$. Phase identification was accomplished by search-match software of ICDD database (International Centre for Diffraction Data, Newtown Square, PA, USA).

Contact angle measurements

Contact angle measurement was used to investigate the wettability of the material surfaces. Cylindrical specimens 10 mm in diameter and 2 mm high were prepared from each material type. A 20 μL drop of distilled water was placed on the surface of the samples and the contact angle was measured by using a contour measurement device comprising a levelled stage, a back-light source and an optical imaging setup. Each experiment was repeated at least three times. The contact angle was measured using image processing and analysis in Java (Imagej) software.

Radiopacity assessment

Three specimens 10 ± 1 mm in diameter and 1 ± 0.1 mm thick were prepared of each material type and were radiographed on a photo-stimulable phosphor (PSP) plate adjacent to a calibrated aluminium step wedge (Everything X-ray, High Wycombe, UK)

with 3 mm increments using a standard X-ray machine with an exposure time of 0.50 seconds at 10 mA, tube voltage at 65 ± 5 kV and a cathode-target film distance of 300 ± 10 mm. The grey pixel value of each step in the step-wedge on the digital images was determined using an imaging program (Adobe Photoshop) and a graph of thickness of aluminium vs. grey pixel value on the radiograph was plotted with the best-fit logarithmic trend line. The equation of the trend line gave the grey pixel value of an object on the image as a function of the object's thickness in mm of aluminium. The grey pixel values of the cement specimens were then determined the relevant thickness of aluminium calculated.

Antimicrobial activity analysis

Three antimicrobial methods were performed: direct contact test (DCT), adenosine triphosphate (ATP) assay and antibiofilm activity by Live/Dead assay and observed using a confocal laser scanning microscope (CLSM). The bacterial strains used were *Streptococcus mutans* ATCC 25175, *Streptococcus gordonii* ATCC 33478 and *Streptococcus sobrinus* ATCC 33399. All bacterial suspensions were prepared in brain-heart infusion (BHI) (Scharlau Chemie S.A., Barcelona, Spain) broth and adjusted using a turbidimeter (Densichek Plus, Biomeriueux, Boston, USA) to match an optical density of the 1.0 McFarland standard. This suspension was diluted 30-fold in broth to obtain a bacterial suspension of approximately 1×10^7 colony forming units per milliliter (CFU/mL). For multispecies bacterial suspensions, these single bacterial suspensions were mixed equally (1:1:1). In the antimicrobial activity tests, all samples were exposed to ultraviolet light for 1 hour for sterilization.

Direct contact test

An area of established dimensions on one side of the wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark) was delimited by measuring two points of the edge of the wells separated by 4 mm in order to ensure the same amount of each material on the vertical wall of the well. The area was coated with each material, with a thickness of 1 mm, using a sterile spatula and a measured using a [calibre \(16\)](#). Once the material was set, samples were exposed to 10-μL of the mixed bacterial suspension for 1 hour at 37°C to ensure direct contact between bacteria and tested materials. Bacterial suspensions placed on the wall of uncoated wells served as the positive control. After incubation 220 μL of sterile BHI was added to each well. The bacterial suspension was mixed for 1 minute, diluted serially and plated for viable cell counting. [Plates were incubated for 24 hours at 37°C under anaerobic conditions.](#) Each group was tested twice, each time in triplicate (n=6/group). [Six wells of each material were inoculated using sterile BHI, as a negative control to check the sterility of the samples.](#) Results of the DCT were expressed as Log₁₀ (CFU + 1/mL).

Antibiofilm activity by ATP assay

Samples 1 mm height and 6 mm diameter (n=6/group) were exposed to 1.8 mL of sterile BHI and 200 μl of the polymicrobial suspension in a 24 microtiter plate incubated on a rocking table (OVAN, model Swing Sw 8 10000-00015, Badalona, Spain), during 7 days at 37°C under anaerobic conditions. The BHI was refreshed every two days. After the incubation period, the disks were rinsed with 0.9% saline solution and the biofilms formed on the materials surface were recovered by placing the disks in eppendorfs with 500 μl of BHI and vortexing for 2 seconds followed by sonication for 10 minutes. The bacteria in the recovered suspension was evaluated by ATP assay. [Six](#)

specimens of each material exposed to sterile BHI were included as negative controls to check the sterility of the samples.

For the ATP assay 100 μ l of the bacterial suspension was added to 100 μ l of the BacTiter Glow reagent (Promega, Madison, WI), according to the manufacturer's instructions, followed by incubation at room temperature for 5 minutes. The luminescence produced was measured with a luminometer (model E6521, GloMax, Promega BioSystem). Luminescence was calculated as the mean of the signals from bacterial culture – the mean of BHI alone and was expressed as RLU (relative light units).

Antibiofilm activity by confocal laser scanning microscopy

Material samples (n=6/group) were exposed to the polymicrobial suspension as described in the previous section. After incubation, the disks were rinsed and stained with Syto-9/Propidium iodide (PI) (Live/Dead, BacLight, Invitrogen, Eugene, OR, USA) (17) for 15 min and were observed under CLSM (Nikon Eclipse Ti-E, Nikon Canada, Mississauga, Canada). Syto-9 is a green-fluorescent stain, labelling both live and dead microorganisms. PI is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes (dead microbes). The excitation/emission wavelengths were 494/518 nm for Syto-9 and 536/617 nm for PI. Six microscopical confocal volumes from random areas were obtained from each sample using a 40 \times oil lens, 1 μ m step-size and a resolution of 512 \times 512 pixels. Each picture represented an area of 317 \times 317 μ m. For quantification purposes the *bioimage_L* software was used (18). The parameters

evaluated in each group were the total biovolume (μm^3) and the percentage of red-stained population (dead cells).

Statistical Analysis

A one-way ANOVA test followed by the Tukey post hoc test were used for comparisons among groups. The data of the red percentages of the CLSM test were previously subjected to the Anscombe transformation as they did not were normally distributed indicated by the Kolmogorov-Smirnov test (19). The level of significance was ($p < 0.05$). Statistical analyses were performed by means of SPSS 20.0 software (SPSS Inc, Chicago, IL).

Results

Material characterization

The surface microstructure of the test materials is shown in Figures 1a and 1b. The light curing resin exhibited a smooth surface with no surface deposits while the filled resins had evidence of hydrating cement particles and surface deposit on them. Theracal showed a rough surface but no deposits. Biodentine had a smooth surface microstructure with deposits of cuboid crystals typical of sodium chloride. Higher power images showed globular surface deposits typical of hydroxyapatite.

The surface XRD scans showed no peaks for resin and resin filled with tricalcium silicate and for Theracal. The radiopacified filled resin exhibited peaks for tantalum oxide on its surface while Biodentine had a strong peak for calcium hydroxide at $18^\circ 2\theta$.

Contact angle measurement

The light curable unfilled resin had a contact angle of $60.3 \pm 3.1^\circ$ and Biodentine also showed an angle of $25.3 \pm 0.8^\circ$ indicating some hydrophobicity. The other materials tested had a contact angle of 0° . The contact angles of the resin and Biodentine were higher than the other materials tested ($p < 0.001$). The resin contact angle was higher than that of Biodentine ($p < 0.001$).

Radiopacity

The results for radiopacity assessment are shown in Figure 1c. The Biodentine exhibited the highest radiopacity compared to the other materials under study ($p < 0.001$). The radiopacified resin-based material (LC-TCS-TO) had a similar radiopacity to the Theracal ($p = 0.741$). The unfilled resin and the resin filled with tricalcium silicate exhibited the lowest radiopacity, which was less than the 3 mm aluminium thickness specified by ISO 6876 (2012) ([20](#)).

Antimicrobial activity analysis

Biodentine showed a significantly higher antimicrobial activity than the rest of the materials in the DCT as no bacterial growth was obtained on its surface (Table 1A). In contrast, its activity was lower when it was exposed to the bacterial suspension for a longer time period in the ATP assay (Figure 2a) and CLSM test (Table 1B). It showed significantly higher RLU and lower dead percentages. A statistically similar activity was observed in LC, LC + TCS, LC + TCS + TO and Theracal in the DCT and ATP assay.

Theracal and the radiopacified resin modified tricalcium silicate-filled material showed less total biovolume than the other materials ($p < 0.05$). This was also matched by a higher dead percentage in these materials and the tricalcium silicate filled resin

when compared to the unfilled resin and Biodentine ($p < 0.05$). Representative CLSM images are shown in Figure 2b.

All negative controls showed no bacterial growth in the different tests.

Discussion

Preserving pulp tissue and avoiding root canal therapy is gaining more importance in modern dentistry. The current study investigates three prototype light curable pulp capping materials and their properties were compared to Theracal and Biodentine. The prototype materials were created by using a resin base which consisted of three resin monomers two of which were (meth)acrylate-based and third cyclic monomer. A tricalcium silicate filler and a radiopacifier, tantalum oxide were added thus creating three prototypes. This was done to assess which of the components was responsible for each property tested. Theracal is also a methacrylate-based resin incorporated with tricalcium silicate and barium zirconate as a radiopacifier (10). Biodentine is water-based and the powder contains tricalcium silicate and zirconium oxide radiopacifier (10). Biodentine exhibited diverse surface characteristics showing material hydration with calcium hydroxide deposits on the surface and the unfilled resin exhibiting a smooth surface morphology. The surface characteristics were linked to the antimicrobial behaviour and biological properties. Both the unfilled resin and Biodentine exhibited some hydrophobicity as evident from the contact angle measurements. This hydrophobicity could affect the microbial activity.

Antimicrobial properties are very important for pulp capping materials. The current study uses various techniques and a multispecies biofilm as opposed to the simple methods such as agar diffusion test and the direct contact test reported in the

1 literature (16) Furthermore, the species were used together rather than tested one
2 species at a time (21,22) making the current experiment more clinically accurate.
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4 Although the microbiota associated with caries is highly diverse (23), mutans
5 streptococci constitute an important part of the dental caries microbiota (24).
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7 Therefore, two mutans streptococci, *S. mutans* and *S. sobrinus*, were included in this
8 study together with a non-mutans streptococci, *S. gordonii*, that is also associated with
9 the dentin carious activity (25).
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18 The DCT was used to assess immediate antimicrobial activity after contact of the
19 microorganism with the material (15), while other antimicrobial tests carried out
20 provided more information on how the materials behave after long exposure times to a
21 bacterial suspension as well as on the biofilm formation on its surface. Testing has
22 shown that Biodentine performs very well at initial contact (in the DCT), similarly to
23 how the material performed in previous research (15), but this antimicrobial activity is
24 not shown in the other tests conducted. In fact, Biodentine lost its antimicrobial activity
25 after prolonged exposure to the biofilm.
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39 The ATP assay was used as the presence of adenosine triphosphate indicates
40 intracellular energy transfer (26) showing that microorganisms are alive if ATP is being
41 produced. Mutans streptococci have been documented to have developed means to
42 alleviate influence of an acidic environment by upregulating proton-translocating F-
43 ATPase to maintain an alkaline environment (27). This might explain why changes in
44 pH may initially disrupt bacterial growth but as a biofilm forms, the microorganisms
45 can adapt to some degree to changes in pH. Biodentine has been reported to have a high
46 pH particularly in the early stages of setting (28). This is caused by the release of
47 calcium hydroxide as part of the hydration reaction (10) which was also shown in the
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current study by the large calcium hydroxide peak in the surface XRD scans. The reduction in pH with time may be the reason for the loss of antibiofilm properties with time in Biodentine. Changes in material chemistry of tricalcium silicate-based materials have been reported in contact with blood (29) and also shown in explanted material retrieved from failed root-end surgery (30) and also material embedded in the subcutaneous tissues of test animals (31). In all cases the main phase on the material surface was calcium carbonate which has a neutral pH. The loss of antimicrobial activity of Biodentine on exposure to a biofilm has been also confirmed in the CLSM Live/Dead staining test, where Biodentine exhibited a significantly higher biovolume and less biofilm killing potential.

Theracal and the other resins that included tricalcium silicate showed lower biovolumes and higher dead percentages indicating higher antimicrobial activity over time. The initial calcium ion release of Theracal is lower than that of Biodentine (10). A previous study that evaluated the antimicrobial activity of 24 hour leachates of these materials showed that at this time the antimicrobial activity is not dependent on the leachates but might be influenced by their pH (15). However, in this study the results in the ATP assay and the CLSM showed that the Theracal and the experimental material exerted a higher antimicrobial activity compared to Biodentine after 7 days of exposure to the bacteria suspension. This higher antimicrobial activity could be related to the toxicity of the unpolymerized monomers release of both after a longer time of exposure (32). The addition of tantalum oxide as a radioopacifying agent does not affect significantly the antimicrobial properties. A recent study showed that the resin type and radiopacifier used affected the material hydration (33). The unfilled resin behaved similarly to Biodentine, indicating that the antibiofilm activity is not due to the resin

1 used. Both Biodentine and the unfilled resin were hydrophobic as indicated in the
2 contact angle measurements.
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6 Hydrophilic surfaces in general have been shown to improved cell adhesion,
7 growth and differentiation compared to hydrophobic surfaces (34). Nonetheless
8 wettability also affects bacterial adhesion, as microbial adhesion strongly depends on
9 the hydrophobic–hydrophilic properties of interacting surfaces (35). In this study
10 Theracal and the tricalcium silicate-filled resins showed similar contact angle
11 measurements, as well as similar antibiofilm activity.
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21 **Conclusions**

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25 The surface characteristics of a material affect its antimicrobial properties. The
26 experimental resin modified materials exhibited comparable antimicrobial properties
27 to other light curable pulp capping agents. Further long term studies on the materials'
28 antimicrobial activity [and their interaction with the dentine tissue](#) are required to
29 assess whether they can potentially result in better clinical outcomes than currently
30 available pulp caps.
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45 **Acknowledgements**

46
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48 This project was financed by Internationalisation Partnership Awards Scheme 2015. Ing
49 James Camilleri of the Department of Metallurgy and Materials Engineering, Faculty of
50 Engineering, University of Malta, Malta for his technical expertise. ERDF (Malta) for the
51 financing of the testing equipment through the project: *"Developing an Interdisciplinary*
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Material Testing and Rapid Prototyping R&D Facility” (Ref. no. 012). Gilles Richard of Septodont France for the Biodentine.

Declaration

I affirm that I/We have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest is disclosed

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Table 1A. Viable counts expressed as mean Log₁₀ (CFU + 1/mL) (standard deviation) of the polymicrobial suspension after being in contact with the materials.

	Log ₁₀
Positive control	4.42 (0.08) ^a
LCS	3.45 (0.20) ^b
LCS+TCS	3.25 (0.32) ^b
LCS+TCS+TO	3.38 (0.19) ^b
Theracal	3.31 (0.35) ^b
Biodentine	0 ^c

Read vertically, different superscript letters indicate statistical differences by Tukey test after ANOVA showed significant values.

Table 1B. Mean of the total biovolume (μm³) and the red (dead) percentage of the bacteria grown on the materials surface after 7 days.

	Total biovolume (SD)	Red percentage (SD)
LCS	30890.80 (24297.67) ^a	26.81 (8.80) ^a
LCS+TCS	33951.40 (21861.43) ^a	43.28 (9.67) ^b
LCS+TCS+TO	12419.50 (7900.10) ^b	50.35 (20.25) ^b

Theracal	13410.25 (7773.48) ^b	49.57 (10.72) ^b
Biodentine	29234.05 (1937.76) ^a	29.94 (18.48) ^a

SD: standard deviation.

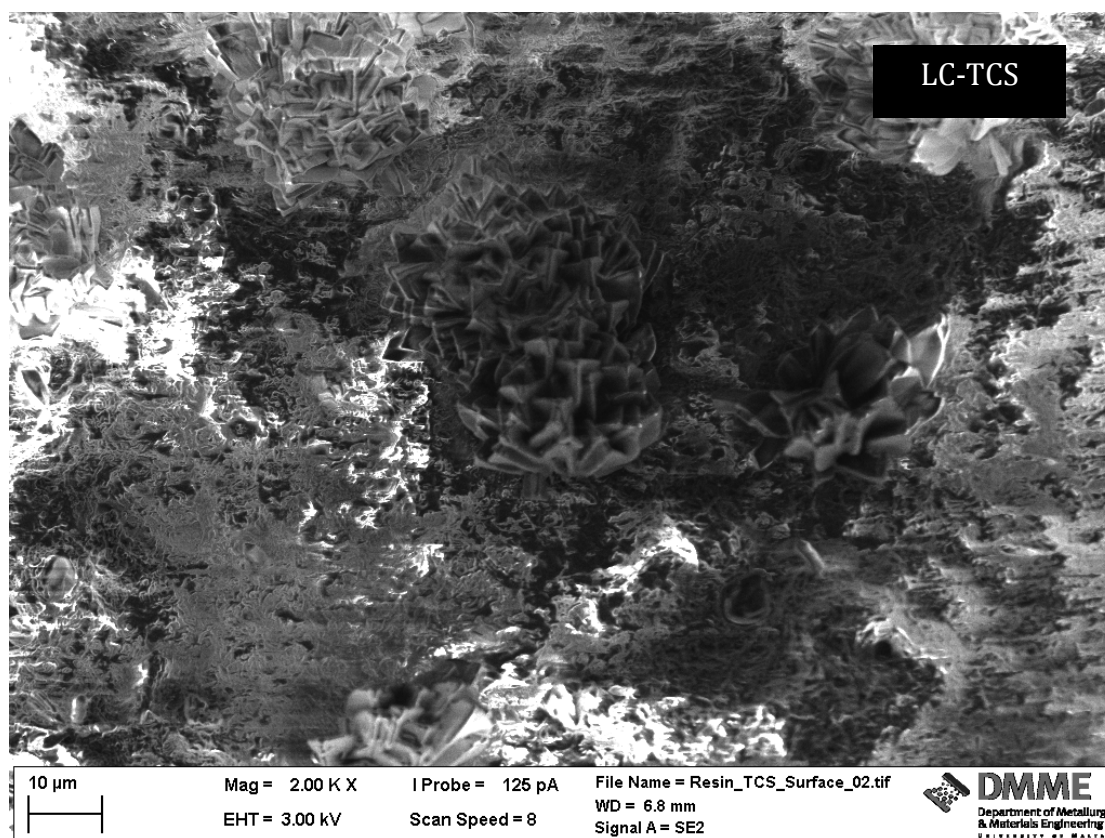
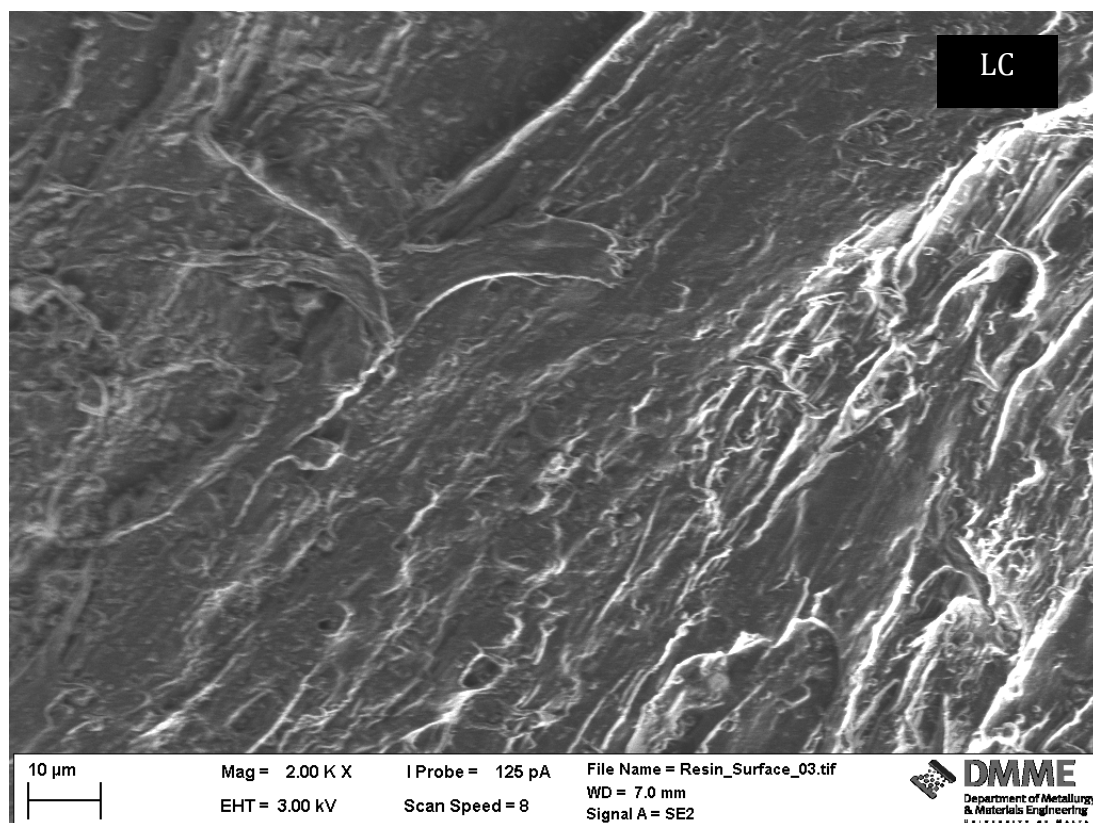
Read vertically, different superscript letters indicate statistical differences by Tukey test after ANOVA showed significant values.

Highlights

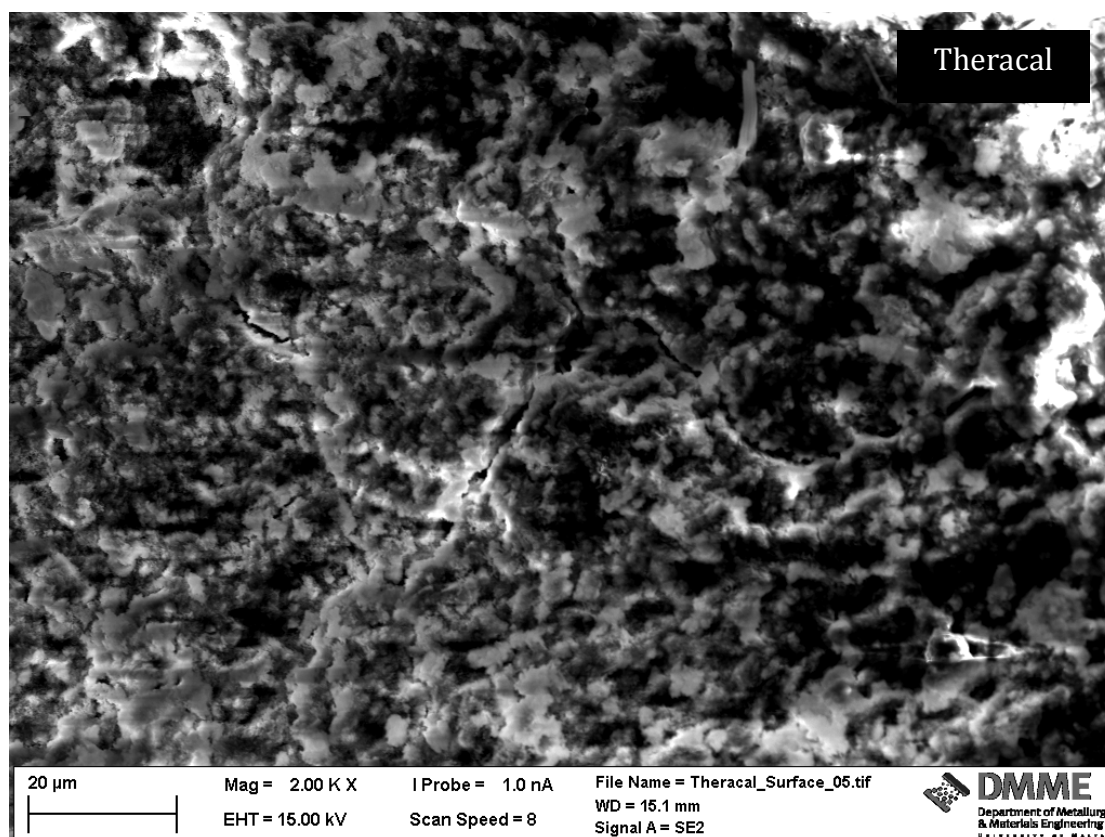
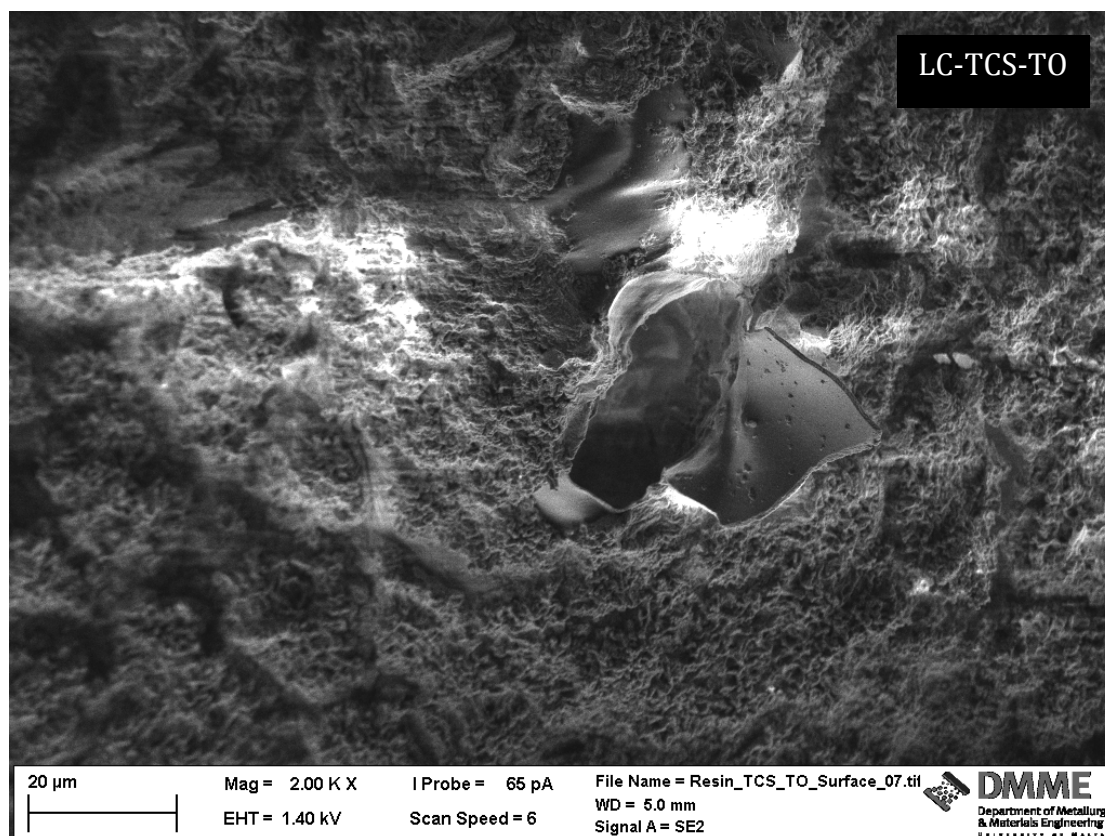
- Light curable pulp capping materials are essential to improve the bonding to overlying resin-based restorations thus reducing micro leakage
- The surface characteristics of a material affect its antimicrobial properties.
- The experimental light curable materials exhibited comparable antimicrobial properties to other light curable pulp capping agents.

Clinical Significance

Materials which possess antimicrobial properties are ideal for pulp capping. The surface characteristics may effect the bacterial attachment.



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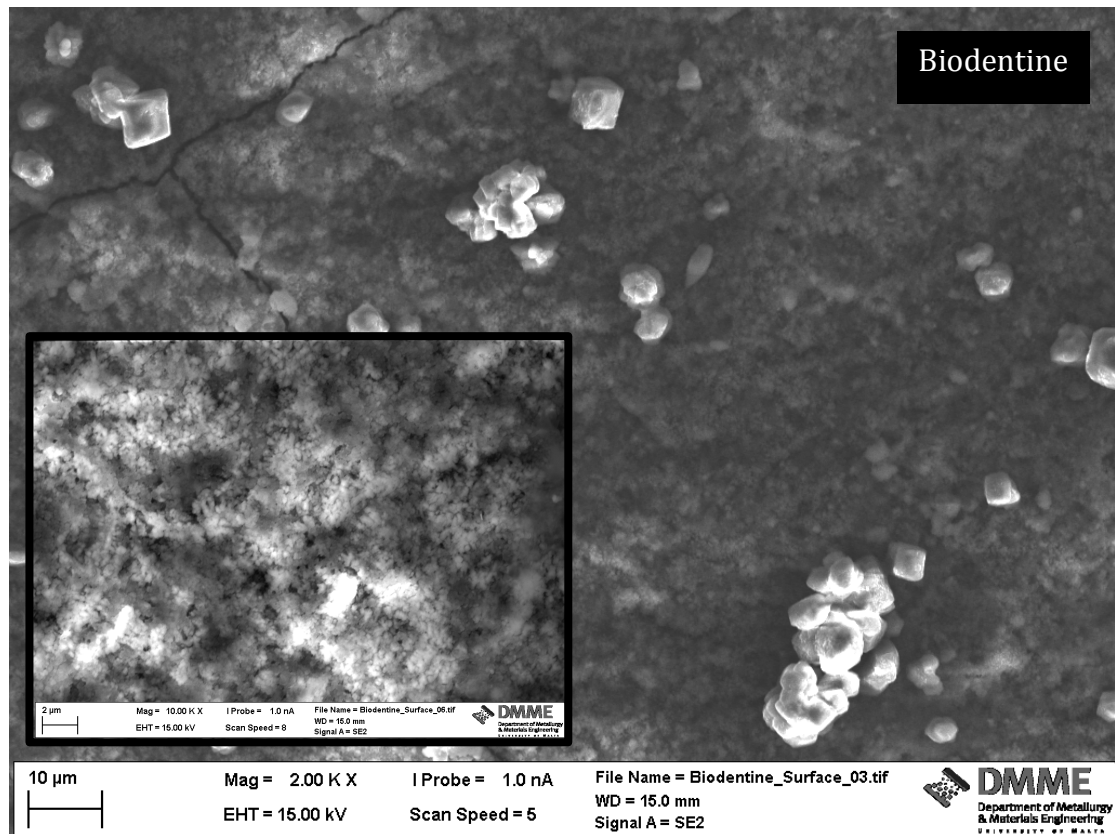


Figure 1a: Secondary electron scanning electron micrographs of test materials showing surface microstructure (Mag: 2K X and inset for Biodentine 10K X)

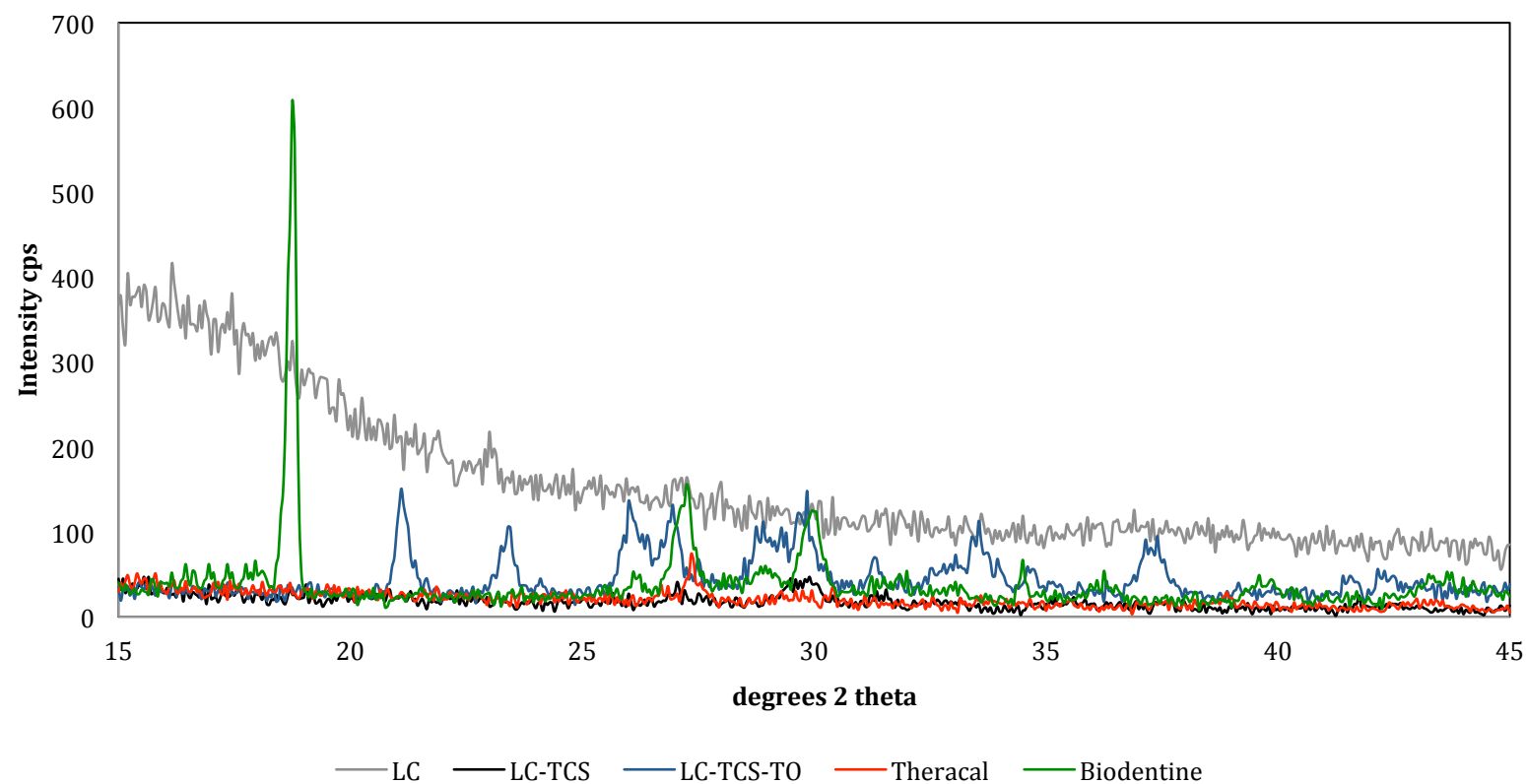


Figure 1b: X-ray diffraction plots of test materials obtained by scanning the material surface at a 3° angle showing the surface constitution of the materials.

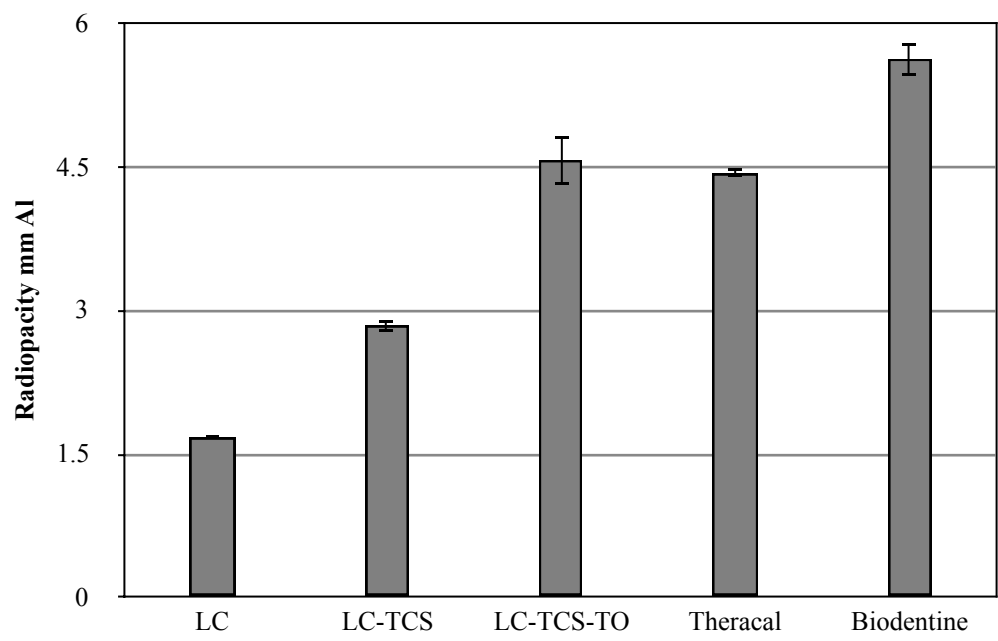


Figure 1c: Mean radiopacity of test materials (\pm SD)

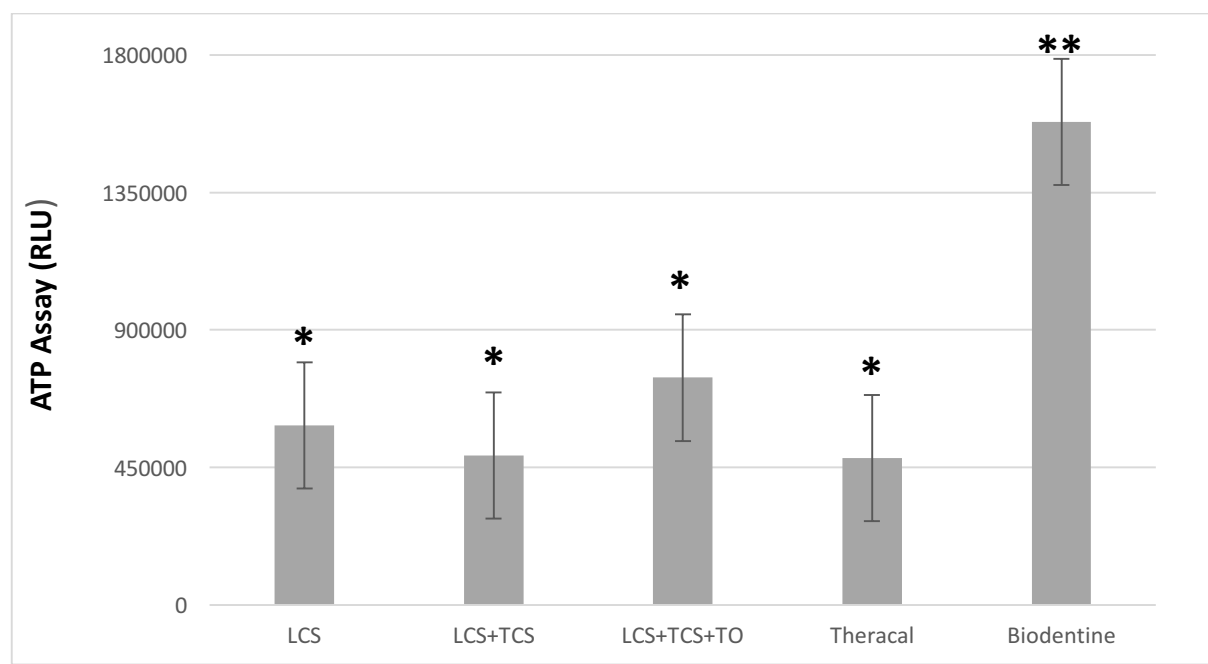


Figure 2a: Values for adenosine triphosphatase activity (ATP) for test materials (\pm SD). Asterisks (*) and double asterisks (**) indicating statistical differences by Tukey test after ANOVA showed significant values.

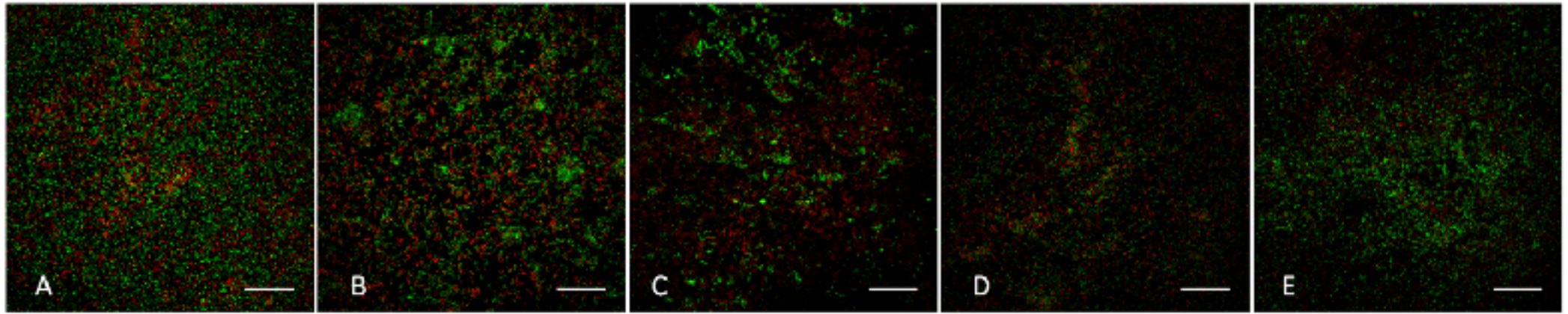


Figure 2b: Representative confocal laser scanning microscope images of bacteria growing on the materials' surface after 7 days: (A) LC, (B) LC + TCS, (C) LC + TCS + TO, (D) Theracal and (E) Biodentine. Bars represent 50 μm .

Reviewer #1: This is a well-written paper that investigates the properties of five pulp capping materials. The study is straight forward, however the Reviewer has few concerns about the methodological approach.

1. Why all the three tested species were streptococci? Literature data showed that in deep carious dentine *Lactobacillus* spp are prevalent.

Reply: *Streptococcus* and *Lactobacillus* species have been considered to be associated with deep carious dentine as well as other species, i.e. *Bifidobacterium* spp.. However, metagenomic studies show that the microbiota associated with caries is highly diverse. In this study we selected 3 streptococci species, 2 mutans streptococci and 1 non-mutans streptococci as they are prevalent in dentine carious lesions and they have also been used in previous studies with multispecies biofilms. A comment on this and a new reference have been included in the Discussion section.

Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011 Mar;90(3):294-303. 5.

Zhang K, Wang S, Zhou X, Xu HH, Weir MD, Ge Y, Li M, Wang S, Li Y, Xu X, Zheng L, Cheng L. Effect of antibacterial dental adhesive on multispecies biofilms formation. J Dent Res. 2015 Apr;94(4):622-9.

Simón-Soro A, Mira A. Solving the etiology of dental caries. Trends Microbiol. 2015 Feb;23(2):76-82.

Arias-Moliz MT, Farrugia C, Lung CYK, Wismayer PS, Camilleri J. Antimicrobial and biological activity of leachate from light curable pulp capping materials. J Dent. 2017 Sep;64:45-51.

2. The experimental setup seems to be useful to test the surface of a material exposed to the oral environment. Nevertheless, it is not the ideal setup to evaluate the anaerobic environment present between infected dentin and composite. Perhaps dentine disks could be a better choice to test the interaction with bacteria. Moreover, the evaluation of an anaerobic environment could be useful to reproduce clinical conditions. The same microorganism shows different behavior in presence of diverse O₂ partial pressure conditions.

Reply: In this study, a first step on the antimicrobial activity of 2 already launched and 1 prototype pulp capping materials was studied by exposing them to a polymicrobial bacteria suspension. We agree with the reviewer's first comment and further research on the interaction between the materials, dentine and the bacteria should be performed.

Regarding the incubation conditions, the reviewer is right and streptococci has been shown to exhibit different behaviours under different environment conditions (oxygen, time of incubation, etc). In fact, a more efficient growth is experienced by the organism with deprived oxygen conditions. In this study, we chose an anaerobic atmosphere to mimic the conditions under restorations where the availability of oxygen is generally very low. This was highlighted further in the manuscript.

Mjör IA. The location of clinically diagnosed secondary caries. Quintessence Int, 1998;29:313-7.

Ahn S, Ahn S, Browngardt CM, Burne RB. Changes in Biochemical and Phenotypic Properties of *Streptococcus mutans* during Growth with Aeration. Appl Environ Microbiol. 2009; 75: 2517–2527.

3. No detailed explanation of sample preparation is present in the text. A table with the description of the materials used in the study should be included in the paper (in particular for the resin-based experimental materials).

Reply: These materials have already been described in other published work. A reference has been added to direct the reader to the description of the materials used.

4. Moreover, the M&M section doesn't include any information about the curing procedures (i.e light source, wavelength, time, distance from the light source). It has been therefore demonstrated that the light

curing procedure is a very important variable in the study of the biological behavior of resin-based materials.

Reply: The curing procedures have been addressed in the Material and Methods section of the manuscript.

5. The description of the specimens preparation is not clear. Authors stated: "An area of established dimensions on one side of the wells of a 96-well microtiter plate (Nunc Delta Surface; Nunc, Roskilde, Denmark) was coated with an equal amount of the materials". The Reviewer can't understand if the material tested was placed on the bottom of the wells (atp test) or on the vertical wall. In the second case, Authors should better describe the procedure and explain how did they determine the area and the thickness of the specimens.

Reply: In the direct contact test (DCT), the materials were placed on the vertical wall of the wells. In order to ensure the equal coating of the sealer, the area of the wall well that was coated was delimited by measuring two points of the edge of the well separated by 4 mm. The materials were placed on this area (from the edge of the well until the beginning of the round bottom) with a thickness of 1 mm, using a sterile spatula and a calibre.

These details have been included in the Material and Methods section.

6. No washing procedure was performed after the polymerization of the resin specimens. This procedure is very important since it has been shown that resins specimens often release unpolymerized monomers both in the biofilm and in the medium, thus influencing the results of the experiment. Authors speculations on the behavior and composition of the biofilm obtained on the specimens surfaces are probably influenced by this relevant bias.

Reply: A previous study that evaluated the antimicrobial activity of 24 hour leachates of these materials showed that at this time the antimicrobial activity is not dependant on the leachates but might be influenced by their pH. However, in this study the results in the ATP assay and the CLSM showed that the Theracal and the experimental material exerted a higher antimicrobial activity compared to Biodentine after 7 days of exposure to the bacteria suspension. This higher antimicrobial activity could be related to the toxicity of the unpolymerized monomers release of both after longer time of exposure. A comment on this and two new references have been included in the Discussion.

Arias-Moliz MT, Farrugia C, Lung CYK, Wismayer PS, Camilleri J. Antimicrobial and biological activity of leachate from light curable pulp capping materials. J Dent. 2017 Sep;64:45-51.

Jeanneau C, Laurent P, Rombouts C, Giraud T, About I. Light-cured Tricalcium Silicate Toxicity to the Dental Pulp. J Endod. 2017 Dec;43(12):2074-2080.

7. How did Authors avoid specimens contamination? No sterilization procedures were described in the M&M section. A microbiological check on the final biofilms seems to be mandatory.

Reply: The reviewer is right and no information regarding the sterilization procedures and the negative controls were mentioned. All samples in the antimicrobial activity tests were exposed to ultraviolet light for 1 hour for sterilization. Negative controls were included in the DCT and in the ATP and CLSM tests to check their sterility. This has been included in the Material and Methods and in the Results sections.

8. SEM microphotographs quality is not excellent. Some overexposure problems occurred.

Reply: The scanning electron micrographs are taken in secondary electron mode to be able to assess the material surface well. The material surfaces are obviously rough as the materials are hand mixed and applied by hand. The resins are non conductive and thus the current quality of the imaging.

9. Why did Authors applied the Anscombe transformation only to CLSM data? How the normality (needed to use ANOVA) of the other sets of data was checked?

1 Reply: In this study, the normality of the data was checked by the Kolmogorov-Smirnov test. In the case of
2 the red percentages obtained in the CLSM, they were not normally distributed and that is why they were
3 previously normalized by the Anscombe transformation. This has been clarified in the Statistical analysis
4 section.
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